

# Mutational Analysis of the Roles of Residues in *Escherichia coli* Elongation Factor Ts in the Interaction with Elongation Factor Tu\*

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The crystal structure of the *Escherichia coli* elongation factor (EF)-Tu-Ts complex indicates that there are extensive contacts between EF-Tu and EF-Ts. To determine the importance of these contacts in the interaction between *E. coli* EF-Tu and EF-Ts, residues in EF-Ts at the interface of these two proteins were mutated. The binding constants governing the interaction of the resulting EF-Ts variants with *E. coli* EF-Tu were determined. The effects of these mutations on the ability of EF-Ts to stimulate GDP exchange with EF-Tu-GDP and on its ability to stimulate the activity of EF-Tu in polymerization were tested. The results indicate that Arg-12, Met-19, and Met-20 in the N-terminal domain of EF-Ts and His-147 and Lys-166 and/or His-167 in subdomain C of EF-Ts are crucial in the interaction between EF-Tu and EF-Ts. Lys-23, Val-234, Met-235, and the C-terminal helix h13 are less important. The binding constants of the EF-Ts variants governing their interactions with EF-Tu correlate well with their activities in stimulating GDP exchange with EF-Tu. Mutations prepared in EF-Tu indicate that His-19 and Gln-114 but not Glu-348 in EF-Tu are moderately important for its interaction with EF-Ts.

Guanine nucleotide-binding proteins play crucial roles in numerous cellular processes (1). These factors cycle through GDP- and GTP-bound states that reflect their active and inactive forms. Many of these proteins require a guanine nucleotide exchange factor to promote the conversion of the GDP-bound form to the GTP-bound form. One of the best studied of the guanine nucleotide-binding proteins is elongation factor Tu (EF-Tu).<sup>1</sup> During the process of polypeptide chain elongation in protein biosynthesis, EF-Tu promotes the binding of aminoacyl-tRNA (aa-tRNA) to the acceptor site (A-site) of the ribosome (2). This binding reaction requires the formation of a ternary complex (EF-Tu-GTP-aa-tRNA). The correct ternary complex is selected through codon-anticodon interactions at the A-site. EF-Tu then hydrolyzes the bound GTP and is released from the ribosome as an EF-Tu-GDP complex (3). A second elongation factor (EF-Ts) promotes the release of GDP forming

an intermediate EF-Tu-Ts complex (4). In *Escherichia coli*, GTP binds to the EF-Tu-Ts complex promoting the release of EF-Ts and the formation of an EF-Tu-GTP complex. This complex binds another aa-tRNA forming the ternary complex again and the cycle repeats.

The three-dimensional structures of EF-Tu complexed to its ligands have been reported (5–9). Analysis of these structures indicates that EF-Tu folds into three domains. Domain I encompasses the first 200 residues and includes the guanine nucleotide-binding site. Domains II and III are each about 100 residues in length. All three domains are required for binding the aa-tRNA (8). EF-Ts can be divided into four structural units as follows: the N-terminal domain (residues 1–54), the core domain (residues 55–179 and 229–263), the dimerization domain (residues 180–228), and the C-terminal module (residues 264–282). The core domain is divided into subdomain N (residues 55–140) and subdomain C (residues 141–179 and 229–263). The crystal structure indicates that there are extensive regions of contact between *E. coli* EF-Tu and EF-Ts (9). The N-terminal domain, subdomain N, and the C-terminal module all interact with domain I of EF-Tu, whereas subdomain C interacts with domain III.

Examination of the three-dimensional structure of the EF-Tu-Ts complex originally suggested that nucleotide exchange arises in part because the side chains of Asp-80 and Phe-81 of EF-Ts intrude into a site on EF-Tu near where the Mg<sup>2+</sup> ion interacting with GDP is normally located (9). The resulting disruption of the Mg<sup>2+</sup> ion binding site was postulated to reduce the affinity of EF-Tu for GDP. However, mutation of Asp-80 or Phe-81 to Ala only results in a 2–3-fold reduction in the ability of EF-Ts to catalyze guanine nucleotide exchange with EF-Tu-GDP (10). The mutation of both Asp-80 and Phe-81 has a more deleterious effect (about 10-fold) on the activity of *E. coli* EF-Ts. These observations suggest that other regions of EF-Ts must be playing a role in promoting GDP exchange and/or in the binding of EF-Ts to EF-Tu. In the present work, we have prepared a number of mutations that alter residues at the interface between EF-Tu and EF-Ts. The effects of these mutations on the affinity of EF-Ts for EF-Tu and on its ability to catalyze guanine nucleotide exchange have been analyzed.

## MATERIALS AND METHODS

**Construction of *E. coli* EF-Tu and EF-Ts Mutants**—*E. coli* EF-Tu and EF-Ts genes were cloned into pET24c(+) (10), and site-directed mutagenesis of individual residues was performed using a polymerase chain reaction-based “linker scanning” method (11). Polymerase chain reaction was also used to construct a derivative of EF-Ts carrying a deletion in helix h13 (residues 271–282).

**Expression and Purification of EF-Tu and EF-Ts**—A His-tagged form of *E. coli* EF-Tu was expressed and purified as described previously (10). A His-tagged form of *E. coli* EF-Ts was purified under two different conditions basically as described previously (10). In the first set of conditions, no GDP was present in the extraction buffers. This procedure was used to analyze the amount of EF-Tu co-purifying with EF-Ts or it mutated derivatives. In the second set of conditions, 10 μM GDP

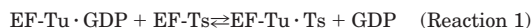
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<sup>1</sup> The abbreviations used are: EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts (The proposed nomenclature for these factors is now EF1α and EF1β, respectively. The traditional nomenclature is being used here for the sake of continuity with the extensive literature on these two factors.); aa-tRNA, aminoacyl-tRNA.

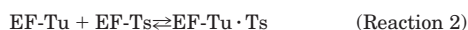
was included in the buffers as described previously (10). This procedure was used to prepare EF-Ts or its variants free of EF-Tu.

**Measurements of Binding Constants and Assays of Activities**—The equilibrium association constants for the binding of GDP to mutated forms of EF-Tu were determined as described previously (12). The binding constants governing the interaction of various mutated forms of *E. coli* EF-Ts to EF-Tu were determined basically as described previously (13). The binding constants governing the interaction of various mutated forms of *E. coli* EF-Tu to wild-type EF-Ts were determined in a similar manner (13). For these experiments, EF-Tu-GDP (0.5  $\mu$ M, 50 pmol), [ $^3$ H]GDP (500 cpm/pmol) (5 or 10  $\mu$ M), and EF-Ts or its variants (1–3  $\mu$ M, 100–300 pmol) were incubated in a reaction mixture (100  $\mu$ L) at 20 °C for 30 min as described (13). The amount of EF-Tu-GDP at equilibrium was measured by the nitrocellulose filter binding assay (14).  $K_{\text{obs}}$  for Reaction 1 is shown below in Equation 1.



$$K_{\text{obs}} = \frac{[\text{EF-Tu} \cdot \text{Ts}][\text{GDP}]}{[\text{EF-Tu} \cdot \text{GDP}][\text{EF-Ts}]} \quad (\text{Eq. 1})$$

$K_{\text{obs}}$  was calculated in each case and used to determine the binding constant ( $K_{\text{Ts}}$ ) for Reaction 2



using the relationship shown in Equation 2.

$$K_{\text{Ts}} = K_{\text{obs}} \cdot K_{\text{Tu-GDP}} \quad (\text{Eq. 2})$$

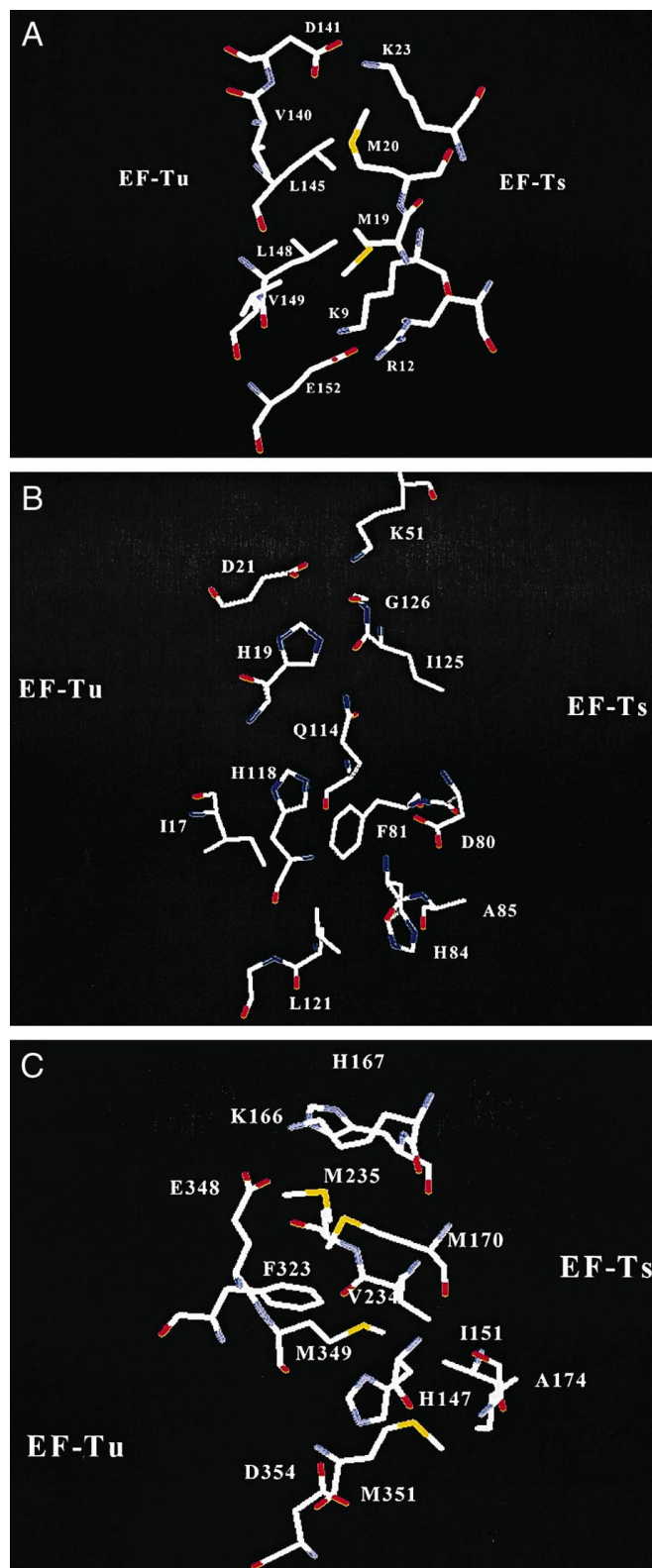
Where  $K_{\text{Tu-GDP}}$  is the binding constant of EF-Tu to GDP ( $3.3 \times 10^8 \text{ M}^{-1}$ ) (12). This value for  $K_{\text{Tu-GDP}}$  was used for experiments using wild-type EF-Tu and for the H19A and E348A derivatives.  $K_{\text{Tu-GDP}}$  was determined to be  $7.9 \pm 2.1 \times 10^7$  for the Q114A variant of EF-Tu. The wild-type EF-Tu and the E348A derivative used for these experiments were about 50% active based on their abilities to bind GDP. The Q114A and the H19A variants of EF-Tu were estimated to be 20% active as estimated by their ability to bind GDP at saturating concentrations of the nucleotide. EF-Ts was estimated to be fully active based on the percentage that could bind to EF-Tu. The activities of EF-Ts and its variants in promoting GDP exchange with EF-Tu-GDP and in stimulating the activity of this factor in poly(U)-directed polymerization of phenylalanine were determined as described (14, 15).

## RESULTS

**Mutational Analysis of EF-Ts Residues Involved in the Interactions between the N-terminal Domain of EF-Ts and Domain I of EF-Tu**—The N-terminal domain of EF-Ts makes a number of contacts with domain I of EF-Tu (Fig. 1A). These interactions include Ala-5, Lys-9, Arg-12, Met-19, Met-20, and Arg-23 of EF-Ts. This area of the interface is bound on the edges by electrostatic and H bond contacts, whereas the interior of the surface is predominantly hydrophobic. This type of distribution of hydrophilic and hydrophobic contacts on the surface of a protein-protein interaction site is quite common (16, 17).

To assess the relative importance of the interactions of these residues in EF-Ts with domain I of EF-Tu, four mutants of *E. coli* EF-Ts (K9AR12A, M19AM20A, M19EM20E, and K23A) were constructed and purified from *E. coli* as His-tagged proteins. When cell extracts are prepared in buffers containing  $\text{Mg}^{2+}$  but lacking GDP, small amounts of EF-Tu are present when wild-type EF-Ts is expressed (data not shown). This level of EF-Tu (about 1 mol of EF-Tu per 10–20 mol of EF-Ts) reflects the relative ability of EF-Ts to compete for binding to EF-Tu with the guanine nucleotides and aa-tRNA present in the cell extract. When cell extracts are prepared in buffers containing GDP, no EF-Tu is present in the preparations of the wild-type factor or any of the mutated forms described below ((10) and data not shown).

The first mutated form of EF-Ts to be tested was K9AR12A. Surprisingly, Lys-9 interacts with EF-Tu primarily through a hydrophobic contact between a side chain  $-\text{CH}_2-$  and Leu-148 in domain I of EF-Tu. Arg-12 makes an electrostatic interaction with Glu-152 and a hydrogen bond with the backbone of



**FIG. 1. Residues of EF-Tu and EF-Ts in contact.** A, residues in the N-terminal domain of EF-Ts contacting residues in domain I of EF-Tu. Arg-12 contacts both Glu-152 and the backbone carbonyl of Asp-109. Only the former interaction is shown for the sake of simplicity. B, contacts between residues in subdomain N of the core of EF-Ts with domain I of EF-Tu. C, residues in subdomain C of the core of EF-Ts in contact with domain III of EF-Tu. Displayed using RASMOL (21). Oxygens are shown in red; nitrogens are shown in purple, and sulfur atoms are shown in yellow.

Asp-109 in EF-Tu (Fig. 1A). No EF-Tu co-purifies with the K9AR12A derivative of EF-Ts (data not shown). This observation suggests that one or both of these residues is important for the interaction of EF-Ts with EF-Tu. To assess the role of these two residues more fully, an attempt was made to measure the binding constant between EF-Tu and the K9AR12A derivative of EF-Ts. These measurements are made by determining the ability of EF-Ts to compete with GDP for binding to EF-Tu (12, 13). The interaction of wild-type EF-Ts with EF-Tu is characterized by a binding constant of about  $9 \times 10^8 \text{ M}^{-1}$  (Table I). The binding constant for the K9AR12A derivative is too weak to be measured in the competition assay used suggesting that there is at least a 100-fold reduction in this value. This mutated form of EF-Ts has no detectable activity in promoting guanine nucleotide exchange with EF-Tu (Fig. 2A) or in stimulating the activity of this factor in poly(U)-directed polymerization of phenylalanine (Fig. 2B).

It is always possible that a mutation resulting in an inactive protein exerts its effect by preventing the normal folding of the polypeptide chain. To assess whether the lack of activity observed with the K9AR12A derivative is the result of the failure of the protein to fold, CD spectroscopy was carried out on wild-type EF-Ts and on the K9AR12A derivative. Spectra were

collected from 178 to 260 nm allowing an assessment of the ability of the mutated protein to fold into the correct secondary structure. The CD spectra of this mutated protein is identical to that of the wild-type protein indicating that it has folded correctly (data not shown).

The observations above indicate that Lys-9, Arg-12, or both of these residues make an essential contact with EF-Tu that is important for the strength of the interaction between these two factors and for the biological activity of EF-Ts. In assessing the relative importance of Lys-9 and Arg-12 in EF-Ts, it should be noted that the major contact between Lys-9 and domain I of EF-Tu is through the  $\beta$ -methylene group that is about 3.8 Å from the side chain of Leu-148. This contact is still available when Lys-9 is mutated to Ala. An examination of the conservation of this residue in EF-Ts from various organisms indicates that Lys-9 is generally a Lys residue in prokaryotes but is Met in mitochondrial EF-Ts. In contrast, Arg-12 is 100% conserved in the EF-Ts' that have been sequenced to date. All of these observations indicate that the lack of activity of the K9AR12A derivative is probably due to the mutation of Arg-12 suggesting that this residue plays a critical role in EF-Ts.

The central core of the interactions between the N-terminal domain of EF-Ts and domain I of EF-Tu is formed by Met-19 and Met-20 which make hydrophobic contacts with Val-140, Leu-145, Leu-148, and Val-149 of EF-Tu (Fig. 1A). They also make backbone hydrogen bonds with residues in domain I of EF-Tu. Although methionine is not universally present at positions 19 and 20 of EF-Ts, these residues are generally hydrophobic as would be expected. When a mutated derivative of these residues (M19AM20A) is prepared and purified from *E. coli*, a small amount of EF-Tu can be seen co-purifying with this protein. In contrast, no EF-Tu is observed in preparations of EF-Ts in which Met-19 and Met-20 have been mutated to glutamic acid. The binding constant between EF-Tu and the M19AM20A mutant of EF-Ts is reduced about 7-fold compared with the wild-type EF-Ts (Table I). No interaction can be detected between EF-Tu and the M10EM20E derivative. This observation is not surprising since the presence of Glu in these positions would completely disrupt the hydrophobic core formed by the interaction of the N-terminal domain of EF-Ts

TABLE I <i>K<sub>obs</sub></i> and <i>K<sub>Ts</sub></i> for <i>E. coli</i> EF-Ts and its mutated derivatives		
EF-Ts	<i>K<sub>obs</sub></i>	<i>K<sub>Ts</sub></i>
		$\text{M}^{-1}$
Wild type	$2.7 \pm 0.4$	$8.9 \pm 1.3 \times 10^8$
K9AR12A	<0.03	< $0.1 \times 10^8$
M19AM20A	$0.37 \pm 0.08$	$1.2 \pm 0.3 \times 10^8$
M19EM20E	<0.03	< $0.1 \times 10^8$
K23A	$2.0 \pm 0.4$	$6.6 \pm 1.3 \times 10^8$
D80A	$1.2 \pm 0.1$	$4.0 \pm 0.3 \times 10^8$
F81A	$0.41 \pm 0.07$	$1.4 \pm 0.2 \times 10^8$
D80AF81A	$0.20 \pm 0.03$	$0.66 \pm 0.10 \times 10^8$
H147A	<0.03	< $0.1 \times 10^8$
K166AH167A	$0.19 \pm 0.05$	$0.63 \pm 0.17 \times 10^8$
V234AM235A	$1.0 \pm 0.2$	$3.3 \pm 0.7 \times 10^8$
V234EM235E	<0.03	< $0.1 \times 10^8$
H13-del	$1.4 \pm 0.3$	$4.6 \pm 1.0 \times 10^8$

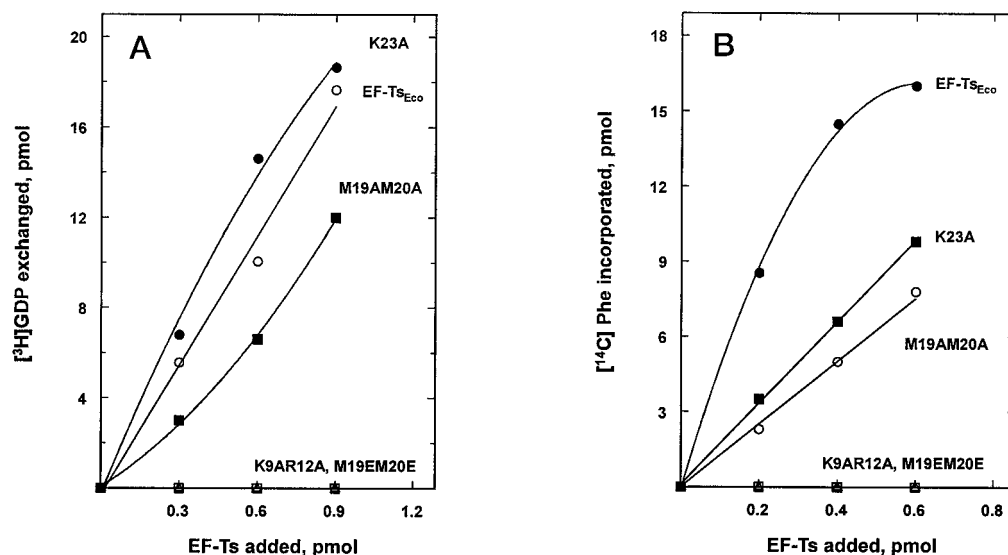


FIG. 2. A, activities of derivatives of EF-Ts carrying mutations in the N-terminal portion of the factor. The stimulation of GDP exchange with EF-Tu was examined as described under "Materials and Methods." Reaction mixtures contained 7.3  $\mu\text{g}$  of expressed EF-Tu (about 80 pmol of active factor) and the indicated amounts of EF-Ts or its mutated derivatives. Blanks representing the amount of GDP exchange carried out by EF-Tu alone during this incubation period (about 7 pmol) have been subtracted from each value. No GDP binding could be detected in the absence of EF-Tu indicating that the preparations of EF-Ts used here were free of EF-Tu. B, stimulation of the activity of *E. coli* EF-Tu in poly(U)-directed polymerization by EF-Ts and its mutants. Reaction mixtures contained 1 pmol of expressed EF-Tu and the indicated amount of EF-Ts. Blanks representing the amount of polymerization catalyzed by EF-Tu alone (about 6 pmol) have been subtracted from each value.



with domain I of EF-Tu. The M19AM20A derivative shows about a 3-fold lower activity in promoting guanine nucleotide exchange with EF-Tu and is about 2-fold less active in stimulating the activity of this factor in polymerization (Fig. 2). As expected, the M19EM20E mutant is completely inactive in these assays. Overall, these observations suggest that Met-19 and Met-20 play a modest role in facilitating the binding of EF-Ts to EF-Tu. Mutation of these residues to Ala creates two small holes in this portion of the interface between EF-Tu and EF-Ts. Loss of some of the hydrophobic binding energy provided by these residues does not have a profound effect on the interaction between these two proteins. However, it is clear that hydrophilic groups cannot be tolerated at this portion of the interface.

The final residue in the N-terminal domain of EF-Ts making contact with EF-Tu is Lys-23. This residue forms an electrostatic contact with Asp-141 and contributes to the hydrophilic edge defining the surface of this region of contact (Fig. 1A). Lys or occasionally Arg are found at this position in the EF-Ts found in various organisms. When a K23A derivative of EF-Ts is purified from *E. coli*, it co-purifies with about the same amount of EF-Tu observed in preparations of the wild-type factor (data not shown). This observation suggests that the K23A derivative of EF-Ts binds to EF-Tu essentially as well as does the wild-type factor. This observation was confirmed by measuring the binding constant of this mutated derivative which is indistinguishable from that of the wild-type factor (Table I). The K23A mutant promotes guanine nucleotide exchange with EF-Tu as effectively as the normal factor and is only about 2-fold less active in stimulating the activity of EF-Tu in polymerization (Fig. 2). These observations indicate that, despite the conservation of a basic residue at position 23, the electrostatic contacts here contribute little to the binding between EF-Tu and EF-Ts.

**Mutational Analysis of Residues Involved in the Interactions between Subdomain N of the Core of EF-Ts and Domain I of EF-Tu**—Subdomain N of the core of EF-Ts makes several potentially important contacts with domain I of *E. coli* EF-Tu (Fig. 1B). The side chains of Asp-80 and Phe-81 are inserted into domain I of EF-Tu near the site where the  $Mg^{2+}$  ion coordinated to GDP is normally located. Asp-80 and Phe-81 are conserved among all of the EF-Ts sequences determined to date suggesting that they play an important role in the activity of this protein. These two residues were mutated in a previous study (10). The D80A and F81A mutants are 2- to 3-fold less active than wild-type EF-Ts in promoting guanine nucleotide exchange. The double mutant D81AF81A is about 10-fold less active. We have now examined the contributions of these two residues to the strength of the interaction between EF-Ts and EF-Tu. As indicated in Table I, the D80A derivative shows only a slightly lower (about 2-fold) binding constant for interaction with EF-Tu. The F81A mutant binds EF-Tu about 6-fold less tightly. The effects of mutation of both these residues are cumulative, and the double mutant (D80AF81A) shows about a 12-fold reduction in the binding constant for EF-Tu (Table I). These values are consistent with the relative abilities of the mutated derivatives to stimulate the activity of EF-Tu.

An additional side chain in subdomain N of the core of EF-Ts (Lys-51) makes an electrostatic contact with Asp-21 in domain I of EF-Tu. This residue has not been as highly conserved as many others and is actually a Leu in the mitochondrial factors that are active in promoting guanine nucleotide exchange with *E. coli* EF-Tu (18, 19). Hence, it is unlikely that Lys-51 plays an essential role in stabilizing the interaction of EF-Tu and EF-Ts. It was not mutated in the present study.

Two contacts are made between backbone atoms in subdo-

TABLE II  
 $K_{obs}$  and  $K_{Ts}$  for *E. coli* EF-Tu and its mutated derivatives

EF-Tu	$K_{obs}$	$K_{Ts}$
		$M^{-1}$
Wild type	$2.7 \pm 0.4$	$8.9 \pm 1.3 \times 10^8$
H19A	$1.0 \pm 0.1$	$3.3 \pm 0.3 \times 10^8$
Q114A	$1.2 \pm 0.1$	$0.95 \pm 0.25 \times 10^8$
E348A	$4.8 \pm 1.0$	$16 \pm 3 \times 10^8$

main N of EF-Ts and side chains in domain I of EF-Tu. A hydrogen bond is found between the carbonyl oxygen of Ile-125 and the amine function of Gln-114. An additional hydrogen bond is found between the carbonyl oxygen of Gly-126 and an imidazole nitrogen in His-19 (Fig. 1B). To examine the roles of these contacts in the interaction between EF-Tu and EF-Ts, two *E. coli* EF-Tu mutants (H19A and Q114A) were created. The H19A derivative of EF-Tu has a binding constant for GDP similar to that of wild-type EF-Tu. This derivative shows a small reduction (about 3-fold) in its binding constant for wild-type EF-Ts (Table II). Wild-type *E. coli* EF-Ts is about 3-fold less active in stimulating GDP exchange with the H19A variant of EF-Tu (Fig. 3A). These observations indicate that contact between the backbone carbonyl of residue Gly-126 in EF-Ts with the side chain of His-19 plays only a minor role in stabilizing the interaction between EF-Tu and EF-Ts.

To assess the role of the contact between the backbone of Gly-126 of EF-Ts and Gln-114 of EF-Tu, a Q114A derivative of EF-Tu was prepared. This derivative has a somewhat weaker binding constant for GDP than the wild-type factor (about  $8 \times 10^7 M^{-1}$  compared with  $3.3 \times 10^8 M^{-1}$ , data not shown). It also has a weaker binding constant for interaction with EF-Ts (about  $0.95 \times 10^8$ , Table II). The reductions in the affinities of this factor for GDP and EF-Ts offset each other to a significant extent. Thus, when wild-type *E. coli* EF-Ts is tested for its ability to stimulate GDP exchange with the Q114A derivative of EF-Tu, it is only 2-fold less active than with wild-type EF-Tu (Fig. 3B). These data indicate that the contact between Gln-114 in EF-Tu and subdomain N of EF-Ts is only modestly important in the interaction between these two proteins.

**Mutational Analysis of Residues Involved in the Interactions between Subdomain C of EF-Ts and Domain III of EF-Tu**—Subdomain C of the core of EF-Ts makes a number of contacts with domain III of EF-Tu (Fig. 1C). These include His-147, Ile-151, Lys-166, His-167, Met-170, Ala-174, Val-234, and Met-235. No analysis of the roles of Ile-151, Met-170, or Ala-174 was carried out in the present work since these residues are not as highly conserved as many others in EF-Ts.

His-147 of EF-Ts interacts with Asp-354 through an ion pair and makes a van der Waals contact with Met-351 in domain III of EF-Tu. His-147 in EF-Ts was mutated to Ala, and the mutated protein was expressed and purified from *E. coli* in buffers lacking GDP. Under these conditions a small amount of EF-Tu co-purifies with wild-type EF-Ts. In contrast, no EF-Tu is detected in preparations of the H147A derivative of EF-Ts. This observation suggests that His-147 may play an important role in the interaction between these two proteins. This idea was confirmed when attempts were made to measure the binding constant for the interaction of the H147A derivative with EF-Tu. The interaction is too weak to be detected suggesting that there is at least a 100-fold decrease in the affinity between these two proteins. A small amount of activity in promoting GDP exchange and in stimulating poly(U)-dependent polymerization can be detected when the H147A derivative is tested (Fig. 4). This observation suggests that this derivative of EF-Ts does indeed have some activity but that its interaction with EF-Tu is significantly impaired. To assess whether the reduced

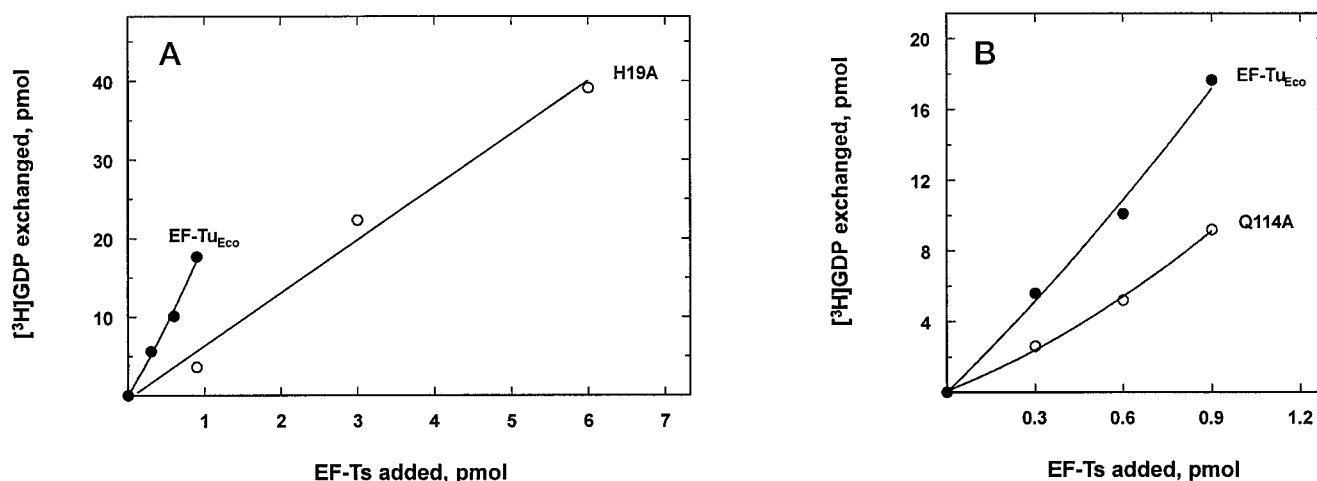


FIG. 3. Stimulation of GDP exchange with wild-type or mutated derivatives of *E. coli* EF-Tu by wild-type EF-Ts. The stimulation of GDP exchange with EF-Tu was examined as described under "Materials and Methods." Reaction mixtures contained the indicated amounts of wild-type EF-Ts and about 80 pmol of active EF-Tu or the H19A variant (A) or the Q114A variant (B). Blanks representing the amount of GDP exchange carried out by EF-Tu alone during this incubation period (about 7 pmol) have been subtracted from each value.

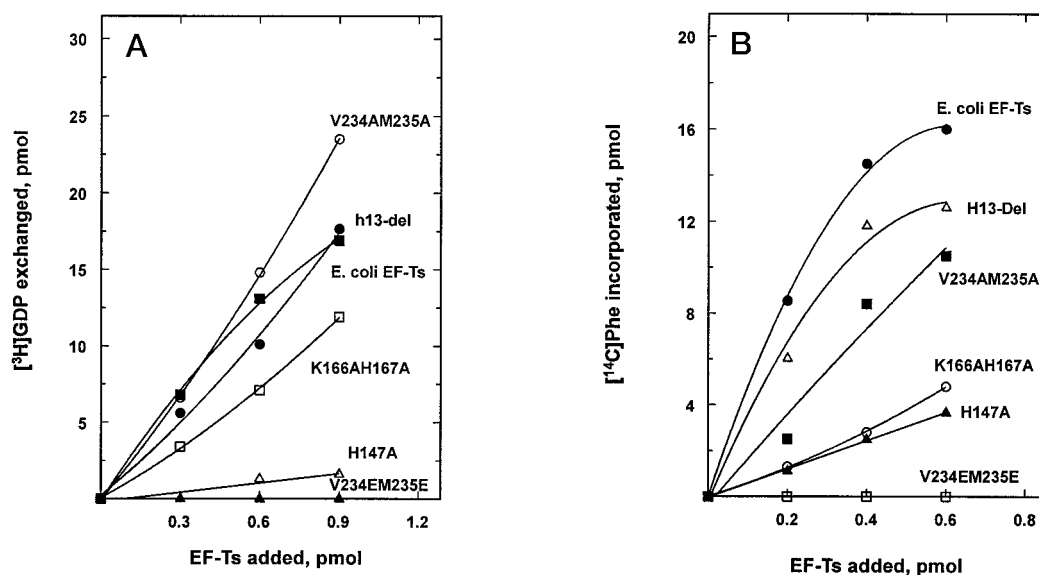


FIG. 4. Stimulation of the activities of *E. coli* EF-Tu by EF-Ts and derivatives mutated in subdomain C. A, the stimulation of GDP exchange with EF-Tu was examined as described under "Materials and Methods." Reaction mixtures contained 7.3  $\mu\text{g}$  of expressed EF-Tu (about 80 pmol of active factor) and the indicated amounts of EF-Ts or its variants. Blanks representing the amount of GDP exchange carried out by EF-Tu alone (about 7 pmol) have been subtracted from each value. No GDP binding could be detected in the absence of EF-Tu indicating that the preparations of EF-Ts used here were free of EF-Tu. B, stimulation of the activity of *E. coli* EF-Tu in poly(U)-directed polymerization by EF-Ts and its mutants. Reaction mixtures contained 1 pmol of expressed EF-Tu and the indicated amounts of EF-Ts. Blanks representing the amount of polymerization catalyzed by EF-Tu alone (about 6 pmol) have been subtracted from each value.

activity observed is due to the failure of most of the molecules in the preparation to fold properly, the CD spectra of this derivative was obtained from 178 to 260 nm and compared with that of the wild-type factor. No differences in these spectra were apparent suggesting that the H147A variant folds like the normal protein (data not shown).

Val-234 and Met-235 of EF-Ts form part of a hydrophobic core in the interaction between EF-Ts and domain III of EF-Tu (Fig. 1C). These residues were mutated to Ala, and the double mutant was expressed and purified from *E. coli*. Significant amounts of EF-Tu co-purify with this variant of EF-Ts suggesting that it binds well. The binding constant between EF-Tu and the V234AM235A derivative is reduced about 3-fold compared with that of the wild-type EF-Ts (Table I). This derivative is also quite active in stimulating GDP exchange with EF-Tu and in promoting the activity of this factor in polymerization (Fig. 4). The replacement of Val and Met residues with Ala would be

expected to cause a reduction in the strength of the hydrophobic interactions in this region of the interface between EF-Tu and EF-Ts. However, replacement of these residues with charged residues would be expected to cause a complete disruption of the interaction. This idea was tested by creating a V234EM235E variant. No detectable binding between it and EF-Tu can be detected (Table I). Finally, the V234EM235E derivative has no activity in stimulating GDP exchange or polymerization with EF-Tu. Taken together, these data argue that a hydrophobic interaction in this portion of the interface is important for allowing the close association of EF-Tu and EF-Ts, but the exact nature of the residues forming this interface is not crucial.

The final region of contact tested between subdomain C of EF-Ts and domain III of EF-Tu is provided by Lys-166 and His-167 in EF-Ts (Fig. 1C). Lys-166 forms an ion pair with Glu-348 of EF-Tu. His-167 makes contact with this same resi-

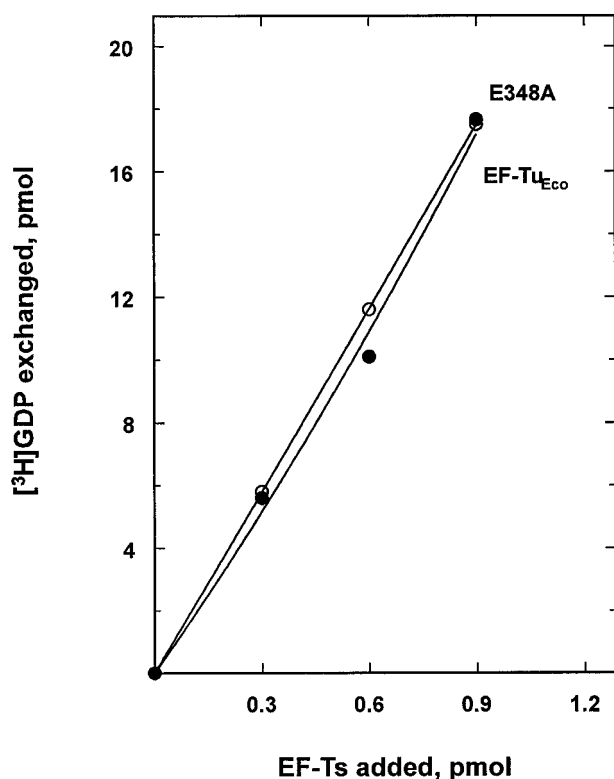


FIG. 5. Stimulation of GDP exchange by wild-type EF-Ts using *E. coli* EF-Tu or its E348A variant. The stimulation of GDP exchange with EF-Tu was examined as described under "Materials and Methods." Reaction mixtures contained about 80 pmol of active EF-Tu and the indicated amounts of EF-Ts. Blanks representing the amount of GDP exchange carried out by EF-Tu alone during this incubation period (about 7 pmol) have been subtracted from each value.

due through a bridging water molecule. Lys-166 is generally a basic residue in the EF-Ts from prokaryotic organisms but is not conserved in the mitochondrial factors. His-167 is not a highly conserved residue. When a H166AK167A variant is purified from *E. coli*, less EF-Tu is observed co-purifying with the mutated protein than with the wild-type EF-Ts. Measurement of the binding constant between the H166AK167A variant and EF-Tu indicates that this derivative has about a 10-fold lower binding affinity for EF-Tu than the normal factor. This variant is 2–4-fold less active in promoting GDP exchange and polymerization with EF-Tu (Fig. 4). These observations indicate that one or both of these residues play a modest role in the interaction observed.

To examine the role of these residues more completely, a complementary derivative of EF-Tu was prepared. This derivative has a mutation that converts Glu-348 in domain III to Ala. The E348A mutant of EF-Tu was purified from *E. coli*. This variant appears to be normal and binds GDP with approximately the same affinity as the wild-type factor (data not shown). Surprisingly, when the E348A mutant of EF-Tu is tested for its ability to bind EF-Ts, it actually has a somewhat higher binding constant than that observed with the wild-type factor (Table II). Wild-type EF-Ts can stimulate guanine nucleotide exchange with the E348A variant as well as with the normal EF-Tu (Fig. 5). This observation seems to be at odds with the reduction in binding constant in the H166AK167A variant of EF-Ts. One possible explanation for this difference may be a reflection of the role of hydrophilic residues at the interfaces of interacting proteins. These residues are often clustered around the outer rim of the interacting surface and serve to protect a series of hydrophobic residues in the core of

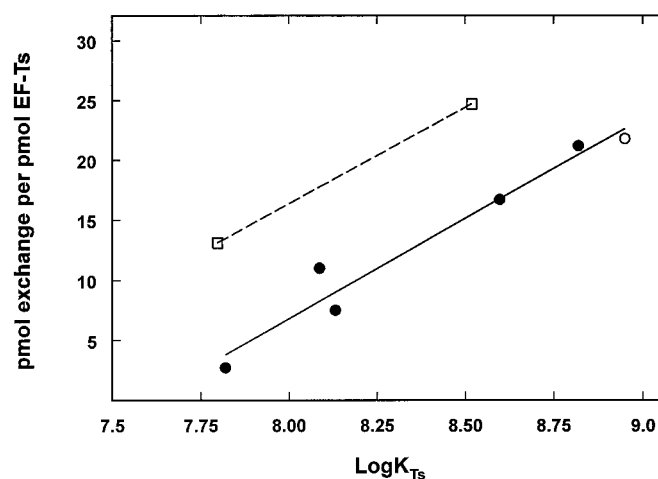


FIG. 6. Relationship between the apparent binding constant for the interaction between EF-Tu and mutated derivatives of EF-Ts and the activity of EF-Ts in promoting guanine nucleotide exchange with EF-Tu. Wild-type EF-Ts (○), mutants in EF-Ts contacting domain I of EF-Tu (●), and mutants in EF-Ts that contact domain III of EF-Tu (□). The dashed line shown for the mutants interacting with domain III should not be taken literally since only two values were available.

the interacting surface from water. His-166 and Lys-167 in EF-Ts may serve this purpose. When both of these residues are mutated to Ala, a hole in the interacting surface may be formed that allows the entry of water molecules. The penetration of water may be sufficient to disrupt the interaction of the hydrophobic residues forming the core of the surface contacts between subdomain C of EF-Ts and domain III of EF-Tu. Mutation of the single residue Glu-348 on EF-Tu may not allow water to enter the hydrophobic core since the side chains of His-166 and Lys-167 may effectively prevent entry of water to this region. Thus, in this interpretation, His-166 and Lys-167 stabilize the interaction of EF-Tu and EF-Ts not through direct contacts but indirectly by protecting the hydrophobic interactions occurring in adjacent parts of the interacting surfaces.

**Deletion Analysis of  $\alpha$ -Helix 13 of EF-Ts**—In the crystal structure of the *E. coli* EF-Tu-Ts complex, the C-terminal  $\alpha$ -helix of EF-Ts folds back across domain I of EF-Tu. This final helical segment is not present in the EF-Ts found in many species including the mitochondrial factors (18). Since mitochondrial EF-Ts can bind *E. coli* EF-Tu and stimulate the activity of this factor, it was of interest to test the importance of helix 13 in the interaction of *E. coli* EF-Tu and EF-Ts. A derivative of *E. coli* EF-Ts (h13-del) was prepared that carries a deletion of the final helix. The binding constant of the deletion derivative to EF-Tu is only about 2-fold lower than the binding constant of wild-type EF-Ts (Table I). The activity of EF-Ts in which helix 13 has been deleted in promoting GDP exchange with *E. coli* EF-Tu is about the same as that of wild-type EF-Ts (Fig. 4A). The activity of the deleted derivative in stimulating the activity of EF-Tu in polymerization is only slightly lower than that of wild-type EF-Ts (Fig. 4B). These observations indicate that the final helical segment in *E. coli* EF-Ts does not play a crucial role in the interaction of this factor with EF-Tu. This observation is compatible with the absence of this sequence in many of the EF-Ts sequences that have been examined to date.

#### DISCUSSION

The binding constants governing the interactions of the mutated variants of *E. coli* EF-Ts with EF-Tu indicate that the contributions of specific residues on the interface to the binding are very different. Whereas residues such as Lys-9/Arg-12,

Met-19/Met-20, Phe-81, His-147, and Lys-166/His-167 are important for the interaction, other residues including Lys-23, Asp-80, and Val-234/Met-235 and helix 13 are not particularly important. More essential contacts appear to occur between the N-terminal domain and subdomain N of EF-Ts with domain I of EF-Tu than occur between subdomain C of EF-Ts and domain III of EF-Tu. Of the residues examined here, Arg-12 and His-147 appear to be the most crucial for the interaction between EF-Tu and EF-Ts.

The structure of the *E. coli* EF-Tu-Ts complex suggested that the mechanism of guanine nucleotide exchange catalyzed by EF-Ts could be explained by the insertion of the side chains of Asp-80 and Phe-81 near the  $Mg^{2+}$  ion binding site in domain I of EF-Tu (9). The loss of the  $Mg^{2+}$  ion which is essential for guanine nucleotide binding would cause the subsequent dissociation of the GDP moiety. However, mutation of these residues individually has only modest effects on the activity of EF-Ts (10). The data reported here indicate that these effects are directly correlated with the moderate decreases in the affinities of these mutated forms for EF-Tu. Analysis of the relationship between the abilities of the mutated forms of EF-Ts to bind EF-Tu and the activities of these derivatives in promoting guanine nucleotide exchange indicates that there is a strong correlation between these values. A semilog plot of  $K_{obs}$  versus activity in guanine nucleotide exchange provides nearly a linear relationship when contacts between domain I of EF-Tu and EF-Ts are considered (Fig. 6). Changes in the contacts between domain III of EF-Tu and EF-Ts appear to be somewhat less serious. The correlation between the binding constant and the nucleotide exchange activity suggests that it may not be reasonable to classify certain residues in EF-Ts as being responsible for catalysis and other residues as being primarily involved in the binding interaction. Rather, the interaction of EF-Ts with EF-Tu is a more global event in which multiple small conformational changes arising from the interaction result in a significant cumulative rearrangement of the guanine nucleotide binding domain of EF-Tu promoting GDP release.

Examination of the crystal structures of the EF-Tu-Ts complex and EF-Tu-GDP indicates that one function of EF-Ts is to increase the separation between domains I and III of EF-Tu (20). This conformational alteration requires interactions of the N-terminal domain and subdomain N of the core of EF-Ts with domain I and EF-Tu and the interaction of subdomain C of EF-Ts with domain III of EF-Tu. The significant structural

rearrangement occurring upon these interactions alters the conformations of a number of loops in domain I that participate in nucleotide binding. Thus, a widespread conformational change propagated through domains I and III of EF-Tu results in GDP release. In this view, any alteration that affects the ability of EF-Ts to interact with EF-Tu will have a subsequent effect on nucleotide release. In the studies reported here, no mutated derivatives of EF-Ts were observed that could bind EF-Tu but that were unable to catalyze GDP exchange. Thus, no single residue or cluster of residues in EF-Ts can be thought of as being responsible for the nucleotide exchange reaction. Residues in this factor important in binding EF-Tu are also important in the nucleotide exchange reaction. A similar situation is likely to be found in the variety of nucleotide exchange factors found in the GTPase superfamily of proteins.

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